resistance mechanisms neutralizing the effectiveness of the therapeutic neurotoxin derivative.

By the same token, the binding moiety may be one other than a binding moiety derived from a clostridial neurotoxin heavy chain, thus providing a targeting function to cell types other than motor neurons.

Also included herein are methods for the construction, expression, and purification of such molecules in high yield as biologically active entities.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A is a diagrammatic view of the single-chain TeTx construct in plasmid pTrcHisA and the nucleotide sequence of the junction region.

Figure 1B shows the and amino acid sequence connecting the carboxyl terminus of the L chain and the amino terminus of the H chain and an engineered loop region containing an enterokinase cleavage site.

Figure 2A is a representation of a Western blot of an SDS-PAGE gel of cell extracts of *E. coli* JM 109 transformants containing 2 different recombinant single-chain toxins, either before or after induction of plasmid protein expression with IPTG. The antibody used for detection is an anti-His_6 monoclonal antibody.

Figure 2B is a Western blot of IPTG-induced cell extracts from cells transformed with the E234A construct.

Figure 3A shows the results of an experiment in which affinity purified recombinant single-chain (SC) TeTx is nicked with enterokinase, then separated using SDS-PAGE and visualized using Coomassie Brilliant Blue under reducing and non-reducing conditions.

single chain holotoxin pro-forms exhibit relatively low toxicity until they are cleaved at one or more peptide bonds in an exposed loop region between their H and L chains to create the fully-active mature neurotoxins. As implied in the mechanism provided above, the H chain of each neurotoxin is essential for cell receptor binding and endocytosis, while both the L and the H chains (and an intact disufide bond) are required for translocation of the toxin into the cytoplasm. As indicated above, the L chain alone is responsible for the toxicity caused by inhibition of acetylcholine secretion.

Despite the clear therapeutic efficacy of clostridial neurotoxin preparations, industrial production of the toxin is difficult. Production of neurotoxin from anaerobic Clostridium cultures is a cumbersome and time-consuming process including a multi-step purification protocol involving several protein precipitation steps and either prolonged and repeated crystallisation of the toxin or several stages of column chromatography. Significantly, the high toxicity of the product dictates that the procedure must be performed under strict containment (BL-3). During the fermentation process, the folded single-chain neurotoxins are activated by endogenous clostridial proteases through a process termed nicking. This involves the removal of approximately 10 amino acid residues from the single-chain to create the dichain form in which the two chains remain covalently linked through the interchain disulfide bond.

The nicked neurotoxin is much more active than the unnicked form. The amount and precise location of nicking varies with the serotypes of the bacteria producing the toxin. The differences in single-chain neurotoxin activation and, hence, the yield of nicked toxin, are due to variations in the type and amounts of proteolytic activity produced by a given strain. For example, greater than 99% of *C. botulinum* type A single-chain neurotoxin is activated by the Hall A *C.*

single chain toxin polypeptide is expressed and purified as above.

Titration of the 6 micrograms of the R496G mutated single chain (WT LC) toxin and the SC TeTx lacking such a mutation against 0, 0.01, 0.1, 1, 10 μg/ml of trypsin, followed by SDS-PAGE and staining with Coomassie Brilliant Blue, yields the cleavage pattern seen in Figure 6. As can be seen, both single chain molecules are susceptible to trypsin cleavage; however the R496G mutant yields fewer fragments than the SC toxin not containing a mutation in the loop region between the chains. For example, while three trypsin peptide bands can clearly be seen near the light chain band upon trypsin cleavage of the SC WT toxin, only two such bands are seen in the R496G digests.

The fact that there exist remaining trypsin sites in the R496G mutant SC toxin probably accounts for the fact that this mutant does not cause the lowering of toxicity as compared to the un-nicked SC toxin; both preparations give similar values in the mouse lethality and neuromuscular paralysis assays described above.

A different assay system is used to measure neurotoxin activity toward CNS neurons, the cells naturally affected by TeTx. The cells used are cerebellar neurons; these cells are disassociated from the cerebella of 7 day old rats. Neurons are suspended at 1-2 x 10^6/mL in medium consisting of 3 parts Basal Eagle Medium and 1 part of a buffer consisting of 40 mM HEPES-NaOH (pH 7.3), 78.4 mM KCl, 37.6 mM D-glucose, 2.8 mM $CaCl_2$, 1.6 mM $MgSO_4$ and 1.0 mM NaH_2PO_4, as well as 1 x N2 supplement, 1.0 mM L-glutamine, 60 units/mL penicillin, 60 μg/mL streptomycin and 5% (v/v) dialysed horse serum. One milliliter of this cell suspension is added to 22 mm diameter poly-D-lysine coated wells. Cytosine β-D-arabinofuranoside (Ara-C, 40 μM) is added after 20-

As shown in Figure 12, the purity of the affinity-purified toxin was demonstrated by SDS-PAGE under reducing conditions, followed by Coomassie staining and Western-blotting, detecting the N-terminus with a mouse monoclonal anti-His antibody from Qiagen (diluted 2000 fold). Enhanced Chemiluminescence solutions (Santa Cruz) and mouse secondary horseradish peroxidase (affinity purified from Sigma) were used for detection of bound antibody. Approximately 2 μg of protein samples were loaded per well.

Example 13

Trypsin activation of Purified Recombinant BoNT/E single-chain Polypeptide.

Purified BoNT/E single-chain neurotoxin polypeptide samples were activated by nicking the single chain with trypsin (1.5 μg/ml final concentration) for 60 minutes at a concentration of 1mg toxin/ml in 10 mm Hepes (pH 7.0), 150 mM NaCl. Following the reaction, the trypsin was inactivated using 0.5 mM PMSF and 10 μg trypsin inhibitor/ml. The quality of the trypsinization was assessed and verified by SDS-PAGE under both reducing and non-reducing conditions, then staining with Coomassie staining and Western blotting the polyacrylamide gel using a mouse monoclonal anti-His antibody (Qiagen, diluted 2000-fold) and a mouse monoclonal anti-H_C IgG (diluted 26-fold). As shown in Figure 13, the Coomassie-stained nicked protein resolves into two bands under reducing conditions, while the heavy and light chains remain disulfide-linked under non-reducing conditions, similar to the native toxin. The antibody-detected recombinant heavy chain is of approximately identical size as its wild-type Clostridium counterpart, whereas the recombinant light chain migrates at a slightly higher molecular weight compared to the native protein.